

**EXHIBIT I**

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rapidly appears to be more complex than that of the steroid hormone receptors, and particularly the glucocorticoid receptor: this is indicated by the observations that (i) the c4T mutant is not defective in ligand-dependent nuclear translocation of the glucocorticoid receptor, which suggests that Arnt is not required for this process (26); and (ii) somatic cell mutants defective in ligand binding or nuclear translocation of the glucocorticoid receptor fall within the same complementation group, which corresponds to the glucocorticoid receptor structural gene (27), whereas analogous mutants for the Ah receptor fall into three complementation groups.

The sequence similarity between the Arnt protein and the *Drosophila* Per and Sim proteins is of interest because Sim is a nuclear protein (21), whereas Per is found in either the nucleus or cytoplasm, depending on the tissue examined (28). The basic helix-loop-helix motif of Arnt is found in certain proteins that bind DNA as homodimers or heterodimers. The motif contains domains for DNA binding and for dimerization (29).

Humans are heterogeneous with regard to the degree of inducibility or the maximally induced activity of cytochrome P450IA1-dependent AHH in their lymphocytes and monocytes. This heterogeneity appears to have a genetic origin, and, furthermore, individuals with high AHH activity appear to be more susceptible to cigarette-induced lung cancer (30). Whether differences in arnt structure, expression, or both underlie this heterogeneity in the human population will be of interest.

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16. PCR was performed on M1-1, which extends from nt 74 to 2616, with the use of an upstream primer with the sequence 5'-CCCGGATCC-3' followed by nt 46 to 117 of the cDNA sequence. The downstream primer contained the sequence 5'-CCCGGATCC-3' followed by the 24 nt of  $\lambda$ gt11 immediately adjacent to the Eco RI cloning site. Both primers therefore contain Bam HI sites. The PCR product was digested with Bam HI and ligated into the Bam HI site of pcDNA-1 (Invitrogen, San Diego, CA) to generate pcDNA-1-M1-1. The insert was excised from pcDNA-1-M1-1 with Sac I and Not I and was ligated to Sac I- and Not I-digested pBMS/NEO (Invitrogen) in order to generate a clone containing the insert in the correct orientation for expression. Colonies containing the insert were analyzed by restriction mapping for the presence of the 95-bp Sac I-Sac I fragment that lies between the cytomegalovirus promoter and the M1-1 insert. The c4T cells were transfected with 20  $\mu$ g of pBMS/NEO or pBMS/NEO-M1-1. Three days later, cells were selected in G418 (400  $\mu$ g/ml), subjected to reverse selection, or subjected to both selections.
17. M3-1 extends from position 1 to position 1935 and was obtained from a  $\lambda$  ZAP library (Stratagene).
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33. We thank B. Lovejoy, S. Eisenberg, S. Crews, and S. Kohtz for help with the sequence analysis; A. Poland and S. Safe for [ $^3$ H]dioxin; R. H. Tukey for human liver mRNA; A. J. Lusis and F. S. Hagen for cDNA libraries; A. J. Watson for advice on the gel retardation experiment; J. B. Fagan for information before publication; A. B. Okey and R. D. Prokipcak for advice; and M. Pastor for typing the manuscript. Supported by NCI grants CA 28868 and CA 16048, Department of Energy contract DE FC03 87ER 60615, a predoctoral stipend (E.C.H.) from Associated Western Universities, a predoctoral stipend (H.R.) from American Cancer Society Institutional Grant IRG-131, and a postdoctoral stipend (B.A.B.) from the California Institute for Cancer Research.

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## Site-Specific Recombination Between Homologous Chromosomes in *Drosophila*

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The ability to mark a cell and its descendants genetically so that the resulting cell clone can be distinguished from neighboring cells facilitates studies in animal biology and development. A method of generating clones by inducing homologous mitotic recombination in *Drosophila* with a site-specific yeast recombinase is described. This method allows for frequent mosaicism after mitotic exchange is induced at predefined sites in the genome.

**M**OSAIC ORGANISMS—THOSE WITH cells of different genotypes in a single individual—have provided insights into such issues as the developmental potential and determination of cell clones, cell lineage relations, and restrictions in development, patterns of cell division, and cell auton-

omy (1). In *Drosophila*, such studies have been greatly aided by the availability of several methods for producing these genetic mosaics. Early in development, mosaics can be produced with the use of mutations that cause chromosome instability or with special ring chromosomes that are inherently unstable (2). Later in devel-

Cy;  $P[>w^{hs}]>75A$  Sb/+ males and  $w^{1118}/w^{1118}$ ; +/+;  $P[>w^{hs}]>75A/P[>w^{hs}]>75A$  females were examined for mosaicism. Attention was limited to the 26 large bristles of the dorsal thorax when a fly was scored as mosaic or nonmosaic. FLP was provided by the  $P[ry^+; hsFLP]2B$  insertion. The numbers of progeny in each class are given, with the corresponding percentages in parentheses. HS, heat shock at 38°C for 60 min.; Y, Y chromosome.

Treatment	Number of progeny					
	Cy <sup>+</sup> (hsFLP)			Cy (−FLP)		
	Sb mosaic	Sb nonmosaic	Sb <sup>+</sup>	Sb mosaic	Sb nonmosaic	Sb <sup>+</sup>
No HS	3 (0.5%)	143 (24.6%)	159 (27.3%)	1 (0.2%)	127 (21.8%)	149 (25.6%)
HS	102 (16.6%)	20 (3.3%)	222 (36.2%)	0	97 (15.8%)	172 (28.1%)

opment, mosaics can be generated with the use of ionizing radiation to induce mitotic recombination between homologous chromosomes (Fig. 1A) (3, 4). I now show that the site-specific recombinase of the yeast 2 $\mu$  plasmid can also induce mitotic recombination in *Drosophila*. This recombination occurs at defined sites (Fig. 1B) with high efficiency and without the lethal and mutagenic consequences of ionizing radiation.

A method for mediating site-specific recombination in *Drosophila* was described previously (5). Flies were transformed with the yeast FLP gene—which encodes a site-specific recombinase—under the control of *Drosophila* heat-shock regulatory sequences (hsFLP). The synthesis of FLP recombinase was subsequently induced when flies carrying hsFLP were subjected to heat shock. Flies were also transformed with an allele of the white eye color gene ( $w^{hs}$ ) flanked by direct repeats of the FLP recombination target (FRT) sequence. When the recombinase was induced by a heat shock, recombination between the FRTs occurred and resulted in the excision of  $w^{hs}$  from the chromosome and the production of an eye with white patches in a pigmented background.

To determine whether FLP could mediate recombination between FRTs located on separate chromosomes, I investigated whether it could produce mosaic patches similar to those induced by ionizing radiation. Flies were generated that were homozygous for the recombination target gene,  $P[>w^{hs}]>75A$ , on the right arm of chromosome 3 [the brackets denote material contained within the  $P$  transposable element,  $>$  indicates the presence and relative orientation of an FRT, and 75A is an isolate number, not a cytological location (6)]. One

of the chromosome homologs was then marked with the more distal dominant marker *Stubble* (Sb) (7); the other homolog carried the wild-type allele (Sb<sup>+</sup>). If FLP is able to mediate recombination between the FRTs on these homologs, then it should produce patches of double mutant (Sb/Sb) and wild-type (Sb<sup>+</sup>/Sb<sup>+</sup>) cells. Cells heterozygous for the dominant mutation and the wild-type allele (Sb/Sb<sup>+</sup>) form bristles that are shorter and thicker than those formed by wild-type (Sb<sup>+</sup>/Sb<sup>+</sup>) cells. Cells homozygous for the mutant allele (Sb/Sb) produce bristles that are extremely short and thick.

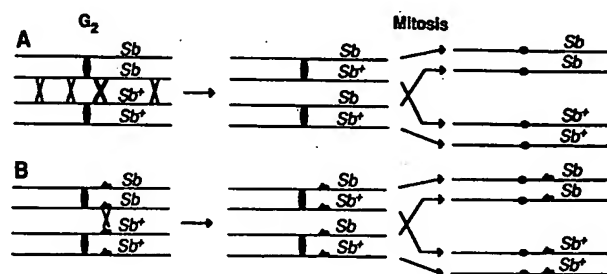
The progeny of the cross shown in Table 1 were examined for such mosaicism (8). Half of these flies received the hsFLP gene on chromosome 2 (hsFLP2B) and the other half received a chromosome 2 that does not carry hsFLP [marked with the *Curly wing* (Cy) mutation]. In each category, half of the flies were homozygous for the recombination target and could be scored for mosaicism because they were heterozygous Sb/Sb<sup>+</sup>. Among the flies that did not carry hsFLP, only a single mosaic was observed. The few mosaics that arise in the absence of FLP almost certainly result from spontaneous mitotic recombination, which occurs with a very low frequency (4, 9, 10). (A decrease in the expected frequency of Sb-

bearing flies was also noted when flies homozygous for the Sb mutation may be slightly deleterious under these conditions.) Flies that did carry hsFLP2B rarely became mosaic in the absence of heat shock. However, after a 38°C heat shock for 60 min during the first 5 to 6 days of development, 84% (102/122) of the potential mosaics became mosaic. Thus, FLP can cause frequent exchange between homologous chromosomes bearing FRT sequences, and this exchange produces the expected mosaics.

To assess the general applicability of this method for generating mosaics, I examined other tissues for FLP-mediated mitotic recombination. In addition to *Stubble* mosaicism, the production of clones homozygous for *multiple wing hairs* (*mwh*) in the wings of flies that were heterozygous for the wild-type and the recessive mutant alleles (*mwh*<sup>+</sup>/*mwh*) (11) was examined. The flies were homozygous for an insertion of the recombinase target ( $P[>w^{hs}]>$ ) proximal to *mwh* on the left arm of chromosome 3. There are ~30,000 cells in each wing that can be scored for the *multiple wing hairs* phenotype, making this a sensitive measure of mitotic exchange. In the absence of FLP, *mwh*/*mwh* clones are rare (12). In the presence of hsFLP2B, and without heat shock, an average of 3.2 clones per wing were produced; the median clone size was one, which means that ~0.01% of the cells were homozygous for the *mwh* mutation. After a heat shock (38°C for 60 min at mid-third instar) ~10.2% of the cells became homozygous. Thus, FLP can also catalyze mitotic recombination in this tissue with high efficiency.

As a third example, I looked for *yellow* ( $y$ ) mosaicism in flies carrying an insertion of  $P[>w^{hs}]>$  proximal to  $y$  on the X chromosome. Homozygous  $y/y$  clones in a heterozygous  $y/y^+$  animal are easily recognized by the resultant yellow bristles. The hsFLP2B gene readily induced such clones on the head, thorax, and abdomen. Therefore, FLP can effect mitotic recombination between

**Fig. 1. Mitotic recombination.** When mitotic recombination occurs in phase G<sub>2</sub> of the cell cycle at a site between the centromere (solid ovals connecting sister chromatids) and a heterozygous marker gene (in this case the *Stubble* locus on the right arm of chromosome 3), the result is homozygous daughter cells after the appropriate type of segregation at mitosis. The alternative type of segregation (not shown) produces heterozygous daughter cells. (A) Exchange induced by x-ray. This exchange may occur at any site along the chromosome, but only events between the centromere and Sb can produce homozygous Sb/Sb and +/+ daughter cells. (B) FLP-mediated exchange. This exchange occurs at the site of integrated FRTs (solid half-arrows). If the FRTs are located between the centromere and Sb, then a single exchange between nonsister chromatids will always allow for the production of homozygous daughter cells.



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somal locations and in a variety of tissues.

I next determined whether FLP could produce mitotic recombination between chromosomes in the male germ line. Recombination in the germ line of male *Drosophila* does not normally occur: recombinant chromosomes are extremely rare, occurring only at a frequency of one in several tens of thousands (13). Germ line recombination was measured in males homozygous for the insertion  $P[>w^{hs}>]75A$  and heterozygous for mutant and wild-type alleles in the flanking loci *mwh* and *Sb* (Table 2). As expected, no recombination was observed in the absence of FLP (Table 2, cross A). A small amount of recombination occurred in *hsFLP2B*-bearing males that were not subjected to heat shock (Table 2, cross B); this *hsFLP* gene typically shows a low level of expression at normal temperatures (5), resulting here in a low level of germ line mitotic recombination without a heat shock. With a heat shock, recombination in the germ line of *hsFLP*-bearing males occurred considerably more often than normal (Table 2, cross B).

Thus, I have shown that FLP can catalyze mitotic recombination in both the soma and germ line. The frequency of recombination can be varied by modulating the severity of the heat shock used to induce FLP synthesis (5). At least two other experimental variables influence the frequency of mitotic recombination: (i) chromosomal position effects alter the activity of *hsFLP* insertions in different tissues; and (ii) the number of FRT sequences at the target sites influences the frequency of recombination for a particular locus.

To assess the effects of chromosomal po-

mosaics were scored as described in Table 1 with  $P[>w^{hs}>]75A$  and  $P[>]75A$  (produced by the FLP-catalyzed excision of  $w^{hs}$ ). FLP was supplied by the *hsFLP1* insertion. The percentage of animals that were mosaic is given together with the total number of relevant progeny scored for each experiment in parentheses. HS, heat shock at 38°C for 60 min.

FRT genotype	Frequency of mosaics (%)	
	No HS	HS
$\frac{>w^{hs}>}{>w^{hs}>}$	97.0 (164)	99.5 (197)
$\frac{>w^{hs}>}{>}$	31.1 (299)	58.5 (325)
$\frac{>}{>}$	1.3 (316)	43.7 (254)
$\frac{>w^{hs}>}{+}$		0.3 (313)

sition on the activity of *hsFLP*, I examined the frequency of recombination obtained at the same target site when *hsFLP* was located at different positions in the genome. In some tests, the differences in recombination frequencies realized with different *hsFLP* insertions were dramatic. For example, when an insertion of *hsFLP* on the X chromosome (*hsFLP1*) was tested, high rates of recombination occurred in the dorsal thorax (as measured by the production of *Stubble* mosaics with  $P[>w^{hs}>]75A$ ) even in the absence of heat shock (Table 3). However, in most instances, the frequency of recombination produced by *hsFLP1*, with or without heat shock, was lower than that produced by *hsFLP2B*. When *hsFLP1* was used, 0.0003% of wing cells were homozy-

gous after heat shock 6.4% of wing cells were homozygous; in the germ line (Table 2, cross C) and in the eye (14), the frequency was also lower. Because the location of the gene that encodes FLP is independent of the location of the recombination event, these chromosomal position effects can provide researchers a convenient method to use in varying the frequency of mitotic recombination in different tissues without heat shock.

FLP-mediated excision of the  $w^{hs}$  gene flanked with a single FRT on each side leaves behind, at the same chromosomal location, a single FRT (5). I used this type of recombination to generate targets at the same chromosomal location with either one or two FRTs. I then measured the rate of mitotic recombination in females with a single copy of the X-linked *hsFLP1* gene and different numbers of FRTs at the same site on each chromosome 3, proximal to *Sb* (Table 3). The frequency of *Stubble* mosaics decreased as the total number of FRTs decreased. This reduction in frequency applies in the male germ line as well (Table 2, cross D). As discussed above, this particular insertion of *hsFLP* expresses the FLP recombinase at a relatively high constitutive level in the tissue used for this assay. In all cases the frequency of mosaics increased after a heat shock was applied to induce higher levels of FLP expression. Nevertheless, as the number of FRTs was reduced, the frequency of mosaics decreased.

This FRT dosage effect implies that each FRT has a certain probability of recombining with an FRT on the homolog. Decreasing the number of FRTs, therefore, decreases the frequency of mitotic recombination. On the other hand, this variation in recombination frequency might also be attributable to a change in chromatin structure after  $w^{hs}$  excision. If the single FRT that remains becomes less accessible to the FLP recombinase, then one might expect to see such a decrease. Of course, these alternatives are not mutually exclusive, and a combination of the two may account for the decreased frequencies of mitotic recombination.

FRTs must be present on both homologs for mitotic recombination; when FRTs were present only on one homolog, the frequency of mosaic production was reduced to background levels (Table 3). Thus, it can be concluded that the FLP-mediated mitotic recombination that produces these *Stubble* mosaics occurs at the FRTs that have been inserted in the chromosome. Sequences that can function as FRTs may occur naturally in the *Drosophila* genome; however, if they exist on the chromosome arms examined (X, 3L, and 3R proximal to *Sb*), they are used far less efficiently by FLP than the FRTs of

**Table 2.** FLP mediates mitotic recombination in the male germ line. The indicated males were test-crossed to *mwh Sb<sup>+</sup>/mwh Sb<sup>+</sup>* females, and the progeny were scored for recombination in the male germ line. Recombinant chromosomes could be scored as *mwh Sb<sup>+</sup>* or as *mwh<sup>+</sup> Sb* progeny. In the heat-shock experiment, the fathers were males that underwent eclosure 8 to 10 days after heat shock; that is, they were subjected to heat shock as young larvae. All were mated on day 10 after heat shock. Cross A, no FLP control; males carried an *S<sup>2</sup>CyO* marker chromosome (7) with no *hsFLP* insertion. Cross B, males carried the *hsFLP2B* insertion. Cross C, males carried the *hsFLP1* insertion. Cross D, males carried, on one homolog, just a single FRT; these males also carried the *hsFLP1* insertion. HS, heat shock at 38°C for 60 min.

Cross	Genotype of male	Treatment	Total progeny	Recombination (%)
A	$\frac{w^{1118}; S^2CyO; mwh}{Y} \frac{P[>w^{hs}>]75A Sb}{+ mwh^+ P[>w^{hs}>]75A Sb^+}$	HS	1136	0.0
B	$\frac{w^{1118}; hsFLP2B; mwh}{Y} \frac{P[>w^{hs}>]75A Sb}{+ mwh^+ P[>w^{hs}>]75A Sb^+}$	No HS HS	707 1171	6.1 37.8
C	$\frac{w^{1118} hsFLP1; mwh}{Y} \frac{P[>w^{hs}>]75A Sb}{mwh^+ P[>w^{hs}>]75A Sb^+}$	No HS HS	395 1128	1.0 30.9
D	$\frac{w^{1118} hsFLP1; mwh}{Y} \frac{P[>w^{hs}>]75A Sb}{mwh^+ P[>]75A Sb^+}$	No HS HS	397 834	0.0 15.1

It has been shown that FLP can excise a chromosomally integrated copy of the *white* gene flanked by directly repeated copies of an FRT. This resulted in somatic and germinal mosaicism for the cloned *white* gene and thus provided a simple and efficient method by which one could make mosaics of cloned genes. My results now extend those findings to show that FLP can mediate exchange between FRTs on homologous chromosomes. With the use of three marker genes (*yellow*, *Stubble*, and multiple wing hairs), I have shown that the mosaicism produced by FLP-catalyzed mitotic recombination can occur in virtually every epidermal tissue and in the germ line of *Drosophila*.

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6.  $P[>w^{1118}>]2A$  and  $P[>w^{1118}>]75A$  are insertions of the *P* element carrying the FRT-flanked *white* gene on the left (L) and right (R) arms of chromosome 3, respectively. The positions of these  $P[>w^{1118}>]$  insertions have been determined by genetic mapping and have been inferred from the observation that they can be used to generate mosaics for genes located on chromosome 3L and 3R, respectively, as described here.  $P[ry^+, hsFLP]$  is a *P* element carrying the *ry*<sup>+</sup> eye color gene and the heat-inducible *FLP* gene;  $P[ry^+, hsFLP]1$  and  $P[ry^+, hsFLP]2B$  are insertions of this element on the X chromosome and chromosome 2, respectively, and are referred to simply as *hsFLP1* and *hsFLP2B*. The construction of these *P* elements has been previously described (5).
7. The mutations used herein have been cataloged and described [D. L. Lindsley and E. H. Grell, *Genetic Variations of Drosophila melanogaster* (Publ. 627, Carnegie Institution of Washington, Washington, DC, 1968)].
8. All flies were allowed to grow at 25°C on standard medium. Heat shocks for all experiments were performed as previously described (5).
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11. For these experiments, females of the genotype  $w^{1118}/w^{1118}; hsFLP2B/+; muh P[>w^{1118}>]2A/muh^+P[>w^{1118}>]2A$  were generated. Wings were mounted and examined at a magnification of  $\times 250$ . For the non-heat shock experiment, pairs of wings from 24 flies were examined. For the heat shock experiment, the left wings from five flies were examined at  $\times 500$ ; in this case, the homozygous *muh/muh* cells were quite numerous, and so, rather than attempt to count separate clones, all the *muh/muh* cells on the dorsal surface of the wing in the region between the third and fourth longitudinal veins and distal to the anterior cross-vein were counted as representative of the entire wing. It has been esti-

the wing, and so to estimate the fraction of homozygous *muh/muh* cells, I divided the median of the five values by 2200.

12. Clones of cells exhibiting the *muh* phenotype arise spontaneously in control *muh/muh*<sup>+</sup> animals that do not carry *hsFLP* or FRTs. The frequency varies somewhat from strain to strain, but is typically between 0.3 [J. Szabad, I. Soos, G. Polgar, G. Hejja, *Mut. Res.* 113, 117 (1983)] and 0.04 clones per wing (9). I observed 0.25 clones per wing in  $w^{1118}/w^{1118}; muh/muh^+$  females. Twelve pairs of wings were examined.
13. R. C. Woodruff and J. N. Thompson, *Heredity* 38, 291 (1977).
14. K. G. Golick, unpublished observations.

nation frequencies in the absence of FRTs were not quantified. However, examination of  $y/y^+$  and *muh/muh*<sup>+</sup> flies that were subjected to heat shock and that carried *hsFLP*, but no homozygous FRT insertions, revealed no obvious increase in the frequency of mosaics relative to background levels.

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## LAV Revisited: Origins of the Early HIV-1 Isolates from Institut Pasteur

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Two of the first human immunodeficiency virus type-1 (HIV-1) strains isolated were authenticated by reanalyzing original cultured samples stored at the Collection Nationale de Culture des Microorganismes as well as uncultured primary material. Cloned polymerase chain reaction products were used to analyze coding sequences of the V3 loop in the gp120 glycoprotein. The original isolate HIV-1 Bru, formerly called LAV, was derived from patient BRU. HIV-1 Lai was derived from patient LAI and contaminated a HIV-1 Bru culture between 20 July and 3 August 1983. The culture became, in effect, HIV-1 Lai, identifiable by a unique motif in the V3 loop. Because of this contamination two, rather than one, HIV-1 isolates were sent to the Laboratory of Tumor Cell Biology at the National Cancer Institute on 23 September 1983. Original HIV-1 Bru was indeed present in the sample marked JBB/LAV. However the M2T-/B sample harbored HIV-1 Lai, a strain capable of growing on established cell lines. The striking similarity between HIV-1 Lai (formerly LAV-Bru) and HTLV-3B sequences remains.

INEVITABLY, PERHAPS, THE ORIGINS OF the first HIV-1 isolates have attracted attention. Lymphadenopathy-associated virus (LAV) was isolated in January 1983 (1). With the agreement on the nomenclature of acquired immunodeficiency syndrome (AIDS) virus isolates, LAV became known as HIV-1 Bru (2). Twelve further HIV-1 isolates were derived by May 1984 from all the high risk groups described at that time. Among the first were HIV-1 DL (3, 4), HIV-1 Lai (4, 5), HIV-1 EL (3, 4), HIV-1 Eli (4, 6), and HIV-1 Ndk (4, 7).

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Together with serological analyses (8) and the realization of the CD4 lymphotropic and cytopathic nature of these viruses (9), these data demonstrated that LAV was the etiological agent of AIDS. The first reports of HIV-1 isolates from the United States were published in the spring of 1984. These included the human T cell lymphotropic virus type 3B, otherwise known as HTLV-3B, from the Laboratory of Tumor Cell Biology (LTCB) at the National Cancer Institute, Bethesda (10-13). LAV-like viruses were also demonstrated in reports from the Centers for Disease Control in Atlanta (14) and the University of California School of Medicine, San Francisco (15).

The molecular cloning of HTLV-3B (16, 17) and LAV (18) was followed by the publication of sequences (19-21). The striking similarity between the two was clearly a separate situation from the divergence usually seen between independent strains, highlighted by the sequence of the HIV-1 SF2 strain (22). Subsequent work on the genetic